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Vision Research 43 (2003) 1513–1517

Vision Research

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Ontogenic changes of kynurenine aminotransferase I activity and its expression in the chicken retina

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Received 4 March 2003; received in revised form 26 March 2003

Abstract

Kynurenine aminotransferases are key enzymes for the synthesis of kynurenic acid (KYNA), an endogenous glutamate receptor antagonist. The study described here examined ontogenic changes of kynurenine aminotransferase I (KAT I) activity and its expression in the chicken retina. KAT I activity measured on embryonic day 16 (E16) was significantly higher than at all other stages (E12, P0 and P7). Double labeling with antibodies against glutamine synthetase showed that on P7 KAT I was expressed in Müller cell endfeet and their processes in the inner retina. Since KAT I activity is high in the late embryonic stages, it is conceivable that it plays a neuromodulatory role in the retina during the late phase of embryogenesis.

Keywords: Kynurenine aminotransferase I; Kynurenic acid; Müller cell; Retinal development

1. Introduction

The tryptophan metabolite kynurenic acid (KYNA) is the only known naturally occurring endogenous glutamate receptor antagonist in the brain (Perkins & Stone, 1982). KYNA in low micromolar concentrations antagonises the glycine site of the NMDA receptor complex (Kessler, Terramani, Lynch, & Baudry, 1989). There is also evidence that KYNA may be a potent noncompetitive antagonist of acetylcholine α7 nicotinic receptors (Hilmas et al., 2001).

It has been suggested that KYNA modulates glutamate-mediated synaptogenesis and neurotransmission during brain development (Ceresoli-Borroni & Schwarcz, 2001). Importantly, changes of KYNA content have been observed in both rat and chicken retinas during ontogeny (Rejdak et al., 2002).

In mammalian cells, KYNA is synthesized by irreversible transamination of L-kynurenine by two distinct kynurenine aminotransferases, KAT I and II (Guidetti, Okuno, & Schwarcz, 1997). KYNA is cleared from the brain almost as quickly as it is formed (Moroni, Russi, Lombardi, Beni, & Carla, 1988; Turski et al., 1988), and only 10% of newly synthesized KYNA is retained in neural tissues; the rest is liberated into the extracellular space (Turski, Gramsbergen, Traitler, & Schwarcz, 1989) where it becomes active and is cleared into the blood stream (Speciale et al., 1990; Turski & Schwarcz, 1988) KATs are highly suitable candidates for biochemical and immunohistochemical investigations (Kapoor, Okuno, Kido, & Kapoor, 1997). KAT I is a soluble enzyme and prefers pyruvate as a co-substrate (Okuno et al., 1990). It has been demonstrated immunohistochemically in the rat brain (Du et al., 1992;

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0042-6989/03/$ - see front matter
doi:10.1016/S0042-6989(03)00233-5

Knyihar-Csillik, Okuno, & Vecsei, 1999; Roberts, Du, McCarthy, Okuno, & Schwarcz, 1992), the medulla, and the spinal cord (Kapoor et al., 1997). KAT II was first identified by Northern blot mRNA analysis in the human brain (Okuno, Nakamura, & Schwarcz, 1991). Only recently, we showed that both KAT I and II were present in the inner retina of the adult rat (Rejdak et al., 2001).

Biochemical analysis of tissue homogenates has shown that enzymatic activity of both KAT I and II is present throughout the brain (Okuno et al., 1991) as well as in the endothelium of vessels (Stazka, Luchowski, Wielosz, Kleinrok, & Urbanska, 2002).

Since avian retinas are avascular (Wolburg, Liebner, Reichenbach, & Gerhardt, 1999), any contamination of retinal samples with KYNA-producing enzymes in such retinas from retinal vessels can be excluded. This makes it possible to examine and qualify the course of KAT I activity during avian retinal development independently of progressive vascularisation.

The present study examined developmental changes of KAT I enzymatic activity and its cellular expression in the chicken retina.

2. Material and methods

All experiments were performed in compliance with the guidelines of animal care in the European Community and the Association for Research in Vision and Ophthalmology.

2.1. Animals

Fertilised White Leghorn eggs and male chickens were supplied by a local hatchery. For tissue preparation, the chickens were deeply anaesthetised with ether between embryonic day 12 (E12) and postnatal day 21 (P21) and decapitated, and the eyes were immediately enucleated. Following hemisection of an eye along the ora serrata the cornea, lens, vitreous body and pecten were removed and the whole neural retinas were then dissected free from the retinal pigment epithelium, choroid and sclera. To assay KAT I activity, retinas were immediately frozen in liquid nitrogen after removal or embedded in cryomatrix for immunohistochemistry.

2.2. Determination of KAT I activity

KAT I activity was assayed according to the method of Guidetti et al. (1997), with modifications. Tissue was homogenized (1:10; *w*/*v*) in 5 mM Tris–acetate buffer, pH 8.0, containing 50 μM pyridoxal-phosphate and 10 mM 2-mercaptoethanol. The resulting homogenate was dialyzed overnight at 8 °C using cellulose membrane dialysis tubing against 4 l of the same buffer. Active samples contained the enzyme preparation with a reaction mixture containing 2 mM L-kynurenine, 1 mM pyruvate, 70 μM pyridoxal-5′-phosphate, 150 mM Tris–acetate buffer pH 9.5 (KAT I), in a total volume of 0.2 ml. Blanks containing heat-deactivated enzyme (100 °C for 10 min) and active samples were incubated at 37 °C for 24 h. The reaction was terminated by immediate transfer of the samples to an ice-bath and the addition of 10 μl of 50% trichloroacetic acid. The denaturated proteins were removed by centrifugation, and the supernatant was applied to the columns containing cation-exchange resin (Dowex 50 W^+: 200–400 mesh) prewashed with 0.1 M HCl. Subsequently, the columns were washed with 1 ml of 0.1 M HCl and 1 ml of water, and the fraction containing KYNA was eluted with 2 ml of water. Eluate was subjected to HPLC, and KYNA was detected fluorometrically (Hewlet Packard 1050 HLC system: ESA catecholamine HR-80, 3 μm, C_{18} reverse-phase column, flow rate of 1.0 ml/min; Hewlet Packard 1046A fluorescence detector: excitation 344 nm, emission 398 nm). L-kynurenine (sulphate salt), KYNA, glutamine, pyruvate, pyridoxal-5′-phosphate, 2-mercaptoethanol and cellulose membrane dialysis tubing were obtained from Sigma (St. Louis, USA). All HPLC reagents were obtained from Baker (Holland).

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test. A *p*-value of less than 0.05 was considered significant.

2.3. Immunohistochemistry

Embedded retinas were radially sliced in 10 μm thick sections, and the sections were mounted on gelatin-coated glass slides. All immunohistochemical incubation steps were carried out on these slides. Endogenous peroxidase was blocked with 3% H_2O_2 in 40% methanol; subsequently, sections were incubated for 1 h with 10% normal goat serum (NGS; Sigma, Munich, Germany) and 0.3 % Triton X-100 in phosphate-buffered saline (PBST) to reduce background staining. The primary antibodies were diluted in PBST containing 10% NGS for immunohistochemistry with antibodies against KAT I and incubated overnight at 4 °C. KAT I was detected using an anti-KAT I polyclonal antibody raised in rabbit against rat kidney KAT (Okuno et al., 1990). A monoclonal antibody directed against glutamine synthetase was used as a marker for Müller cells (Chemicon, dilution 1:500).

Antibodies were diluted 1:50 in PBST. After washing with PBS, the samples were incubated for 1 hour with the biotin-conjugated secondary antibody (dilution 1:200; Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) in PBST with 5% NGS. After rinsing in PBS, retinal sections were processed with an avidin–biotin–peroxidase complex (Vectastain Elite Kit; Vector

Laboratories), and staining was visualized with diaminobenzidine reaction products. For double-labeling immunofluorescence, the preblocking step and primary antibody incubation were performed in 20% NGS plus 2% bovine serum albumin (BSA, Sigma). The secondary antibodies were conjugated either to Alexa 568 rabbit, and Alexa 488 mouse (bought from Molecular Probes; dilutions were 1:1000).

3. Results

3.1. KAT I activity

KAT I activity in 12-day-old (E12) and 16-day-old (E16) embryonic retinas was 0.88 ± 0.1 $(n = 4)$ and 2.1 ± 0.27 $(n = 4)$ (pmol/mg protein, mean ± SE) respectively. At P0 it reached a concentration of 1.1 ± 0.35 $(n = 5)$ and stayed at a comparable level (1.06 ± 0.2, $n = 5$) at P7. The KAT I activity in E16 was significantly higher $(p = 0.043)$ than at other stages (Fig. 1).

Chromatograms showed a peak identical to that obtained for commercially available KYNA (data not shown).

3.2. Immunohistochemistry

KAT I immunoreactivity was concentrated in the inner retina. The most prominent staining was found below the cell bodies of the ganglion cell layer up to the retina-vitreous border. In addition, labeling was present in processes ascending from this brightly stained band into the inner plexiform layer (IPL) and up to innermost row of the inner nuclear layer (INL) (Fig. 2(A)).

Double labeling with KAT I antibody (Fig. 2(A)) and glutamine synthetase antibody (Fig. 2(B)) revealed a clear co-expression of both antibodies. Double labeling was especially prominent in the endfeet of Müller cells and in the processes surrounding the cell bodies in the ganglion cell layer (GCL). It was also found, although less distinctly, along the distal margin of the KAT I immunoreactivity in the INL (Fig. 2(C)).

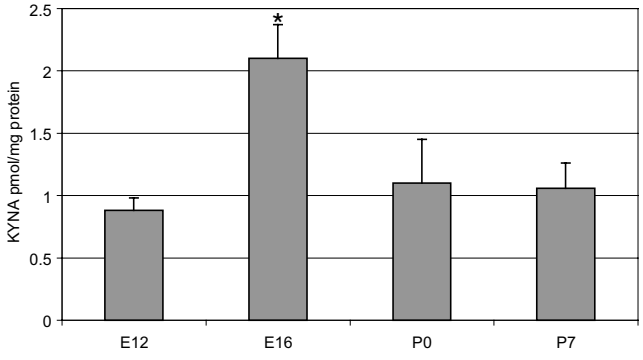

Fig. 1. KAT I activity at different developmental stages (E12–P7) of the chicken retina. Each column represents the mean ± SE (pmol/mg protein). KAT I activity at E16 is significantly higher ($^*p < 0.05$) than at all other stages.

4. Discussion

The experiments in the present study demonstrated that KAT I activity is already present in the chicken retina at early stages of ontogeny. KAT I activity measured at E16 was higher by 58% than at E12 and higher by 48% than at P0. KAT I activity measured at P 7 was more or less at the same level as at P0. Since it has been proven that KAT I activity contributes to the formation of KYNA (Guidetti et al., 1997), these data correspond to our previous findings showing that in the chicken retina KYNA concentrations were significantly higher at E16 than at E12 and rapidly decreased to adult levels by postnatal day 7. In the rat retina KYNA content peaked at birth, when it was 7 times higher then at E20 and then decreased during the first two weeks of life (Rejdak et al., 2002).

It has been suggested that changes of KYNA availability might modulate the function of excitatory synapses (Carpenedo et al., 2001). In the immature brain, NMDA receptors are crucial for the modulation of neuronal migration (Komuro & Rakic, 1993) and for synapse development (Schwarcz, Poeggeler, Rassoulpour, Ceresoli-Borroni, & Hodgkins, 1998). Since glutamate receptors are already present at birth and show differences in spatial distribution and temporal expression in both chicken (Somohano, Roberts, & Lopez-Colome, 1988) and rat retinas (Grunder, Kohler, & Guenther, 2000; Grunder, Kohler, Kaletta, & Guenther, 2000), it is possible that marked changes of KAT I activity in the pre- and post-hatching period, such as those observed in the present study, lead to the previously reported changes of retinal KYNA metabolism (Rejdak et al., 2002) and modulate glutamate-mediated synaptogenesis and neurotransmission in the retina. Since it has been suggested that KAT I plays a role in the regulation of programmed cell death (Csillik, Okuno, Csillik, Knyihar, & Vecsei, 2002) it is conceivable that KAT I and KYNA may control apoptosis in the retina during early development.

Glutamate has been shown to diminish KYNA synthesis in brain slices, and its regulatory influence on endogenous KAT activity has been suggested (Urbanska, Chmielewski, Kocki, & Turski, 2000). An increase in retinal glutamate content occurs as glutamatergic cells mature in other species (Haberecht & Redburn, 1996) and it is possible that the decrease in retinal KAT I activity might result from a similar changes in glutamate level in chick retinas.

Kynurenate formation is controlled by the availability of L-kynurenine (Turski et al., 1989) and the

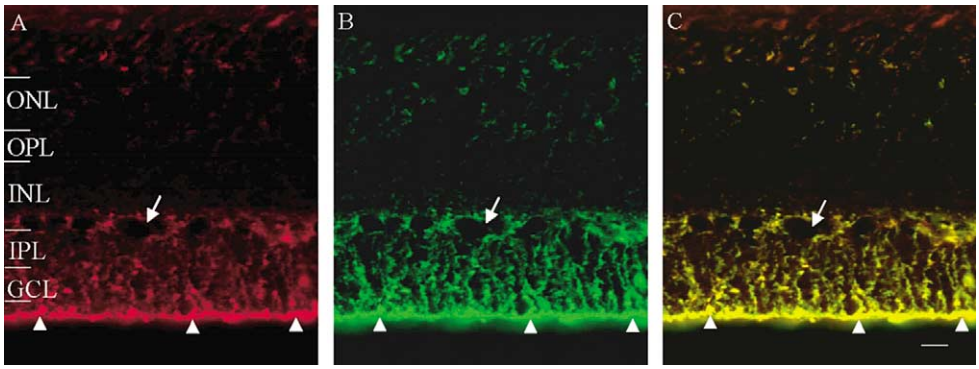

Fig. 2. Localization of KAT I in the chicken retina at P7: (A) positive immunoreaction of KAT I antibody in radial sections of the chicken retina; triangles mark the brightly stained band along the retina-vitreous border and an arrow shows an unstained cell body in the innermost row of the INL; (B) positive immunoreaction of glutamine synthetase antibody (triangles and arrow mark the same structures as in A) and (C) immunostaining in A (red) and B (green) was merged; yellow color indicates the co-localization of both antibodies in the endfeet and ascending processes of Müller cells. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μm.

activities of KAT (Guidetti et al., 1997). Interestingly, studies on age-related changes of KAT activity in the rat brain showed a marked heterogeneity of enzymatic changes between brain regions. A significant increase of KYNA production in the cortex, the hippocampus and the striatum during ageing was observed, while in the thalamus and the cerebellum changes were much less pronounced in adult rats and a decrease was even found in older animals (Gramsbergen, Schmidt, Turski, & Schwarcz, 1992). Taking into consideration the results of the present study, it is conceivable that age-related changes of KYNA synthesis are specific for brain structures.

Double-labeling studies performed in P7 showed that KAT I is localized in Müller cell endfeet at P7 in the chicken retina. This is in agreement with earlier data which suggest that KAT I is preferentially expressed in Müller cell endfeet in the adult rat retina (Rejdak et al., 2001). However, the immunohistochemistry was performed at only one time point. It is possible that the cell-type expressing KAT I could change as the retina develops. In the rat hippocampus, KAT I immunoreactivity was observed mainly in glial cells (Du et al., 1992), while in the striatum it was found in both glial cells and neurons (Roberts et al., 1992). Predominantly neuronal localization of KAT I was observed in the spinal cord (Kapoor et al., 1997).

5. Conclusion

KAT I is present in the avascular chicken retina during ontogeny and is localized in Müller cell endfeet. Since KAT I enzymatic activity is high in late embryonic stages it is conceivable that it plays a neuromodulatory role in the chicken retina during the late phase of embryogenesis.

Acknowledgements

The study was supported by Fortüne Grant 994 of the Medical Faculty of the University Tübingen and the European Union under the Marie Curie Individual Fellowship to Dr. med. Robert Rejdak (QLK2-CT-2002-51562).

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